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The Identity and Phylogeny of *Pseudanabaena* Strain, NIES-512, Producing 2-methylisoborneol (2-MIB)

Akihiro Tuji* and Yuko Niiyama

Department of Botany, National Museum of Nature and Science,
Amakubo 4–1–1, Tsukuba, Ibaraki 305–0005, Japan
* E-mail: tuji@kahaku.go.jp

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Abstract A *Pseudanabaena* strain, NIES-512, has a morphological character that is intermediate between *P. foetida* and *P. subfoetida*. Although the 16S rRNA shows very high similarity among these species, the secondary structures of the 16S rRNA-23S rRNA spacer region were different between NIES-512 and these two species. Because of the morphological similarity and high similarity of the 16S rRNA region, we assigned these differences to the variety level. *P. foetida* var. *intermedia* Tuji et Niiyama is here newly described based on NIES-512, and a new combination *P. foetida* var. *subfoetida* is proposed.

Key words: 2-MIB, *Pseudanabaena foetida* var. *intermedia*, *Pseudanabaena foetida* var. *subfoetida*, secondary structure.

Introduction

A *Pseudanabaena* strain, NIES-512 maintained at the National Institute for Environmental Studies (NIES), was isolated from Nagoya castle, Nagoya, Aichi Prefecture, Japan by N. Yamada on 1 November 1981 as *Phormidium tenue* Gomont (Watanabe *et al.*, 2000). It was re-identified by T. Honma as *Pseudanabaena galeata* Böcher, and is now distributed by NIES using this identification (Kasai *et al.*, 2009). Since the strain produces a musty-odor compound, 2-MIB, it has been used in a great deal of research (Yamada *et al.*, 1985; Kasai *et al.*, 2009; Iwase and Abe, 2010) and has become a model strain for 2-MIB producing cyanobacteria.

Recently, we described two new *Pseudanabaena* species that produce 2-MIB (Niiyama *et al.*, 2016). Ecological characters are important on species level (Anagnostidis and Komárek, 1988; Komárek and Anagnostidis, 1999). *Pseudanabaena galeata* is first observed on the muddy bottom of shallow blackish lake (Böcher, 1949) and it is epiphytic or endogloeic (Komárek and Anaganostidis, 2005). Böcher (1949) describes that *P. galeata* is able to slowly move. Niiyama *et al.* (2016) point out the morphological similarity of *P. foetida* and *P. galeata* and differences of their habitat and motility. The identification of NIES-512 needed to be re-examined considering the morphology, habitat, motility and genetic characteristics. In this paper, we clarify the identity and phylogeny of NIES-512.

Materials and Methods

Cultured strains, genomic DNA extraction, PCR amplification and sequencing

The strains were provided by NIES and maintained under the conditions described in Niiyama *et al.* (2016). The methods of micro-photographing and fixing followed those used by Niiyama *et al.* (2016), and information on other strains was also obtained from the previous report. The

methods of genomic DNA extraction, PCR amplification and sequencing, were also followed those used by Niiyama *et al.* (2016).

Secondary structure models of ITS regions

The putative secondary structures of the 16S rRNA-23S rRNA spacer region were predicted with the Mfold Web server (Zuker, 2003), with default settings including folding temperature set at 37℃. Four parameters of constraint for Mfold, were set for D1-D1', RNA-Ile, RNA-Ala, and Box-B regions.

Results and Discussion

The micro-photographs of NIES-512 and related taxa are presented in Fig. 1. The cell sizes of NIES-512 were intermediate between those of *Pseudanabaena foetida* Niiyma, Tsuji et Ichise (strain PTG) and *P. subfoetida* Niiyma et Tuji (strain Ak1318). The cell width of NIES-512 was 1.6–2.2 μm and thicker than that of PTG (1.0–1.5 μm) and thiner than that of Ak1318 (2.1–2.9 μm). These sizes also overlapped. The cell lengths of NIES-512 was 3.0–8.3 μm, almost identical to that of Ak1318 (2.5–8.5 μm) and slightly shorter than that of PTG (3.9–11.0 μm). The cell lengths of these strains also overlapped (Table 1). The color of the trichomes and cells was bright blue-green in all three strains. Other morphological characteristics were also shared among these three strains (Table 2).

The phylogeny of 16S rRNA revealed similarity between NIES-512 (LC153790) and two new species described in Niiyama *et al.* (2016). NIES-512 and dqh15 (JF429939.1) are almost identical. Differences were found near the 5′ and 3′ ends, and these might simply be sequencing errors.

NIES-512 and PTG have differences of 5 base positions (0.3% of 1467 bp), and NIES-512 and Ak1318 have differences of 12 base positions (0.8% of 1467 bp). These similarities showed that NIES-512 should be included in the *P. foetida* – *subfoetida* species complex.

Niiyama *et al.* (2016) divided this complex

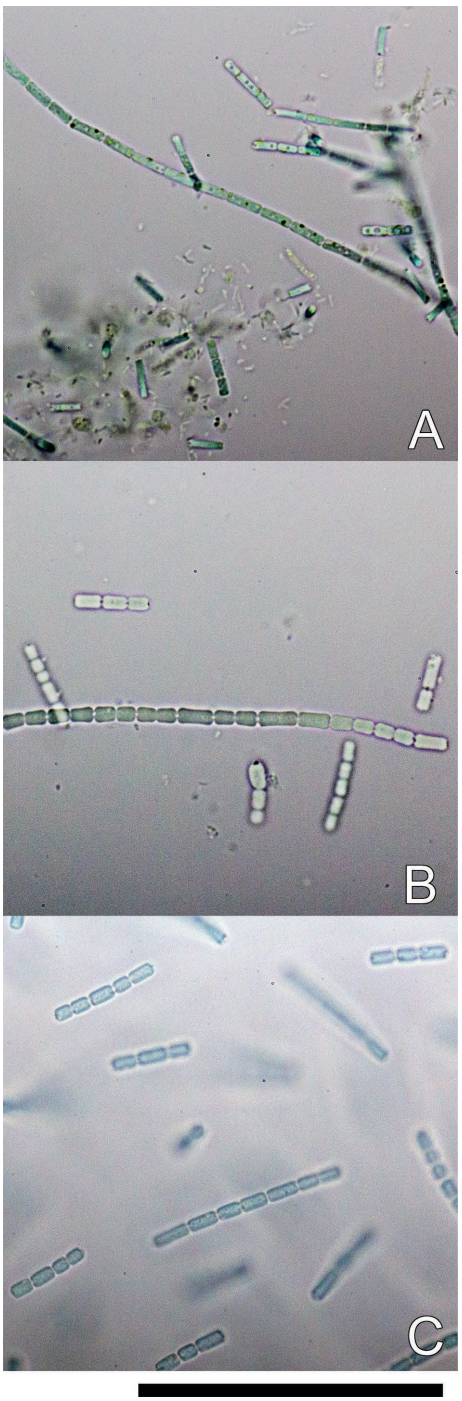

Fig. 1. Micro-photographs of holotype specimens. Scale bar = 50 μm. A. *Pseudanabaena foetida* var. *foetida* (TNS-AL-57781 from PTG). B. *Pseudanabaena foetida* var. *subfoetida* (TNS-AL-58650 from Ak1318). C. *Pseudanabaena foetida* var. *intermedia* (TNS-AL-58658 from NIES-512).

Table 1. Cell size of cultured strains PTG, Ak1318, NIES-512, NIVA-CYA276/6, PTB and Ak1319

Taxon	Strain	Cell width		Cell length		L/W		Specimen no.	Accession number
		min–max μm	mean μm (std.)	min–max μm	mean μm (std.)	min–max	mean (std.)	TNS-AL	16S rRNA
Pseudanabaena foetida var. *foetida*	PTG (Ak1200)	1.0–1.5	1.28 (0.153)	3.9–11.0	6.18 (1.803)	2.7–8.5	4.90 (1.477)	57781	LC016773
P. foetida var. *subfoetida*	Ak1318	2.1–2.9	2.42 (0.204)	2.5–8.5	4.61 (1.391)	0.9–4.0	1.94 (0.664)	58650	LC016779
P. foetida var. *intermedia*	NIES-512 (Ak1363)	1.6–2.2	1.92 (0.138)	3.0–8.3	5.09 (1.255)	1.6–4.6	2.65 (0.677)	58658	LC153790
P. sp.	NIVA-CYA276/6 (Ak1189)	2.0–3.0	2.52 (0.282)	3.0–8.0	5.35 (1.489)	1.0–3.6	2.15 (0.666)	58651	LC016776
P. limnetica	PTB (Ak1201)	0.9–1.3	1.03 (0.131)	2.5–7.1	4.47 (1.276)	2.5–7.0	4.39 (1.269)	57780	LC016774
P. limnetica	Ak1319	1.1–1.5	1.38 (0.113)	1.5–5.0	3.20 (0.947)	1.1–4.2	2.34 (0.735)	58649	LC016775

Cell width and length (μm). L/W = cell length/cell width; std = standard deviation; N = 50.

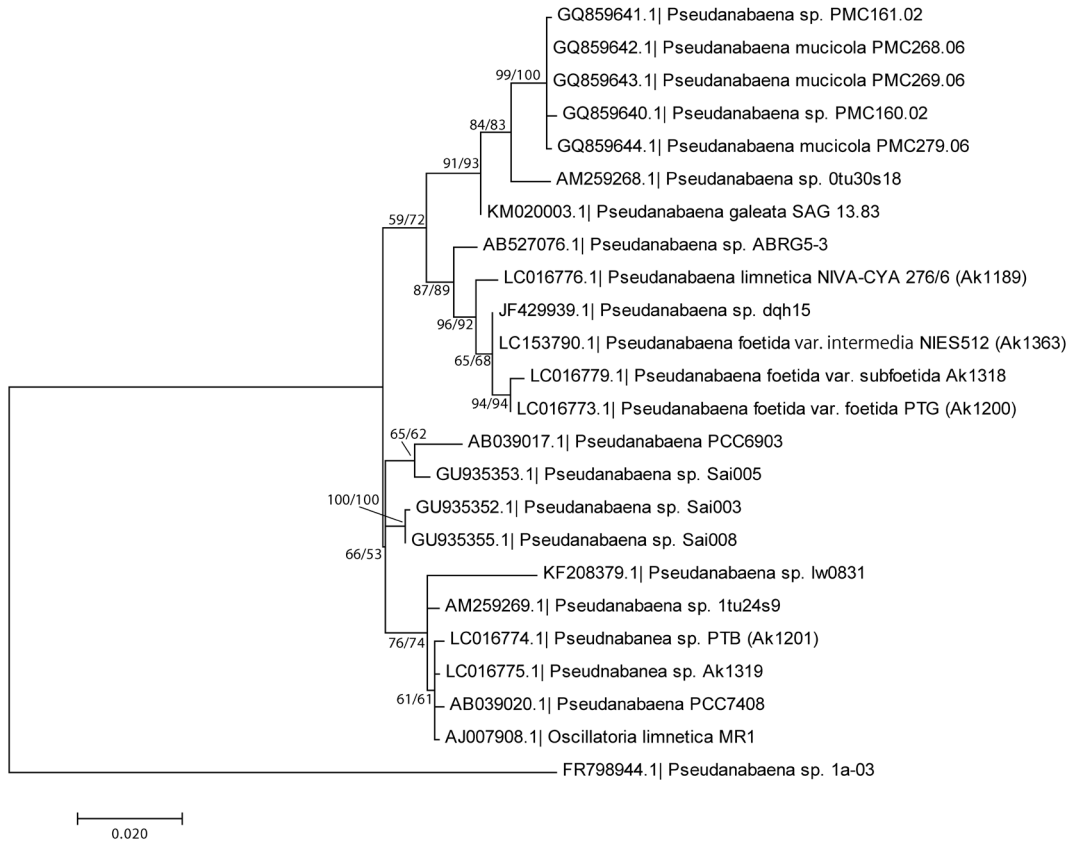

Fig. 2. Phylogenetic position of strains PTG, Ak1318, NIES-512 and related strains determined by Maximum Likelihood (ML) method using 16S rRNA gene. Accession numbers are followed by taxonomic names. Numbers at branches indicate NJ (Neighbor Joining) / ML bootstrap support values (only values higher than 60 are shown).

into two species, *P. foetida* and *P. subfoetida*, based on the secondary structure of the 16S rRNA-23S rRNA spacer region. Since the secondary structures of RNA-Ile and RNA-Ala, presented in Niiyama *et al.* (2016), have problems, four constraint for the D1-D1', RNA-Ile, RNA-Ala, and Box-B regions were set in the Mfold software. The secondary structures of the D1-D1', RNA-Ile, V2, RNA-Ala and Box-B regions were clearly recognized in these three strains (Fig. 3). The structures of RNA-Ile and BOX-B are almost identical within these three strains. The structure of the D1-D1' region in NIES-512 agree with the structure of Ak1318 and differ from that of PTG. The structure of the V2 region in NIES-512 agrees with that of PTG. A long insertion (53 bp) in the V2 region is also identical between NIES-512 and PTG, and does not exist in Ak1318. Several secondary structural differences were seen between NIES-512 and the other two strains. NIES-512 should be in a different taxon from *P. foetida* (PTG) and *P. subfoetida* (Ak1318) based on the secondary structure of the 16S rRNA-23S rRNA spacer region. The secondary structure of this region has been widely used to recognize the taxon (Iteman *et al.*, 2000; Boyer *et al.*, 2001; Siegesmund *et al.*, 2008). However, the 16S rRNA region shows very high similarity among these three strains (>99% similarity). Also, the morphologies of these three strains are very similar, and their dimensions mostly overlap. These three strains commonly

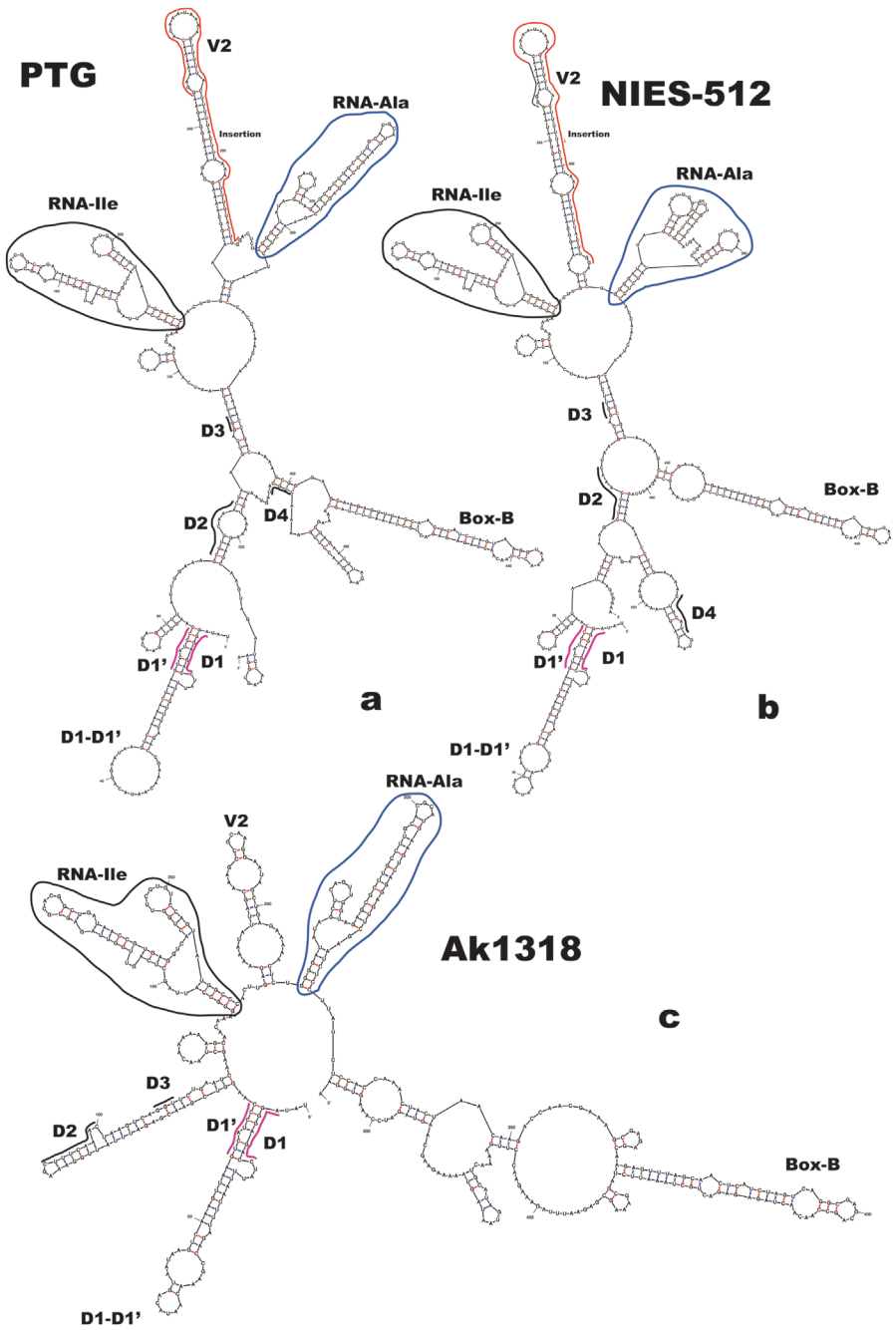

Fig. 3. Secondary structure of the 16S rRNA-23S rRNA spacer region. a. Strain PTG. b. Strain NIES-512. c. Strain Ak1318.

produce 2-MIB. Because of these similarities and to avoid superfluous taxonomic confusion in applied environmental biology, we will divide these strain at the variety level on the basis of their differences.

Table 2. Morphological characteristics of strains, PTG, Ak1318 and NIES-512

Taxon Strain	*Pseudanabaena foetida* var. *foetida* PTG (Ak1200)	*P. foetida* var. *subfoetida* Ak1318	*P. foetida* var. *intermedia* NIES-512 (Ak1363)
trichome color	bright blue-green	bright blue-green	bright blue-green
sheath	—	—	—
motility of trichome	rarely trembling	rarely trembling or rarely slowly go forward	rarely trembling
cell color	bright blue-green	bright blue-green	bright blue-green
cell morphology	long and thin cylindrical	isodiametric to longer than wide	cylindrical
apical cell	long cylindrical with rounded end	isodiamtetric to cylindrical with rounded end	cylindrical with rounded end
poler aerotope	+	+	+
constriction at cross-wall	+	+	+
musty odor	+	+	+

Taxonomic description

Pseudanabaena foetida Niiyama, Tuji et Ichise in Niiyama *et al.*, Fottea **16**: 4-5. *f. 6*, *7*, *14*. 2016. (Fig. 1A)

Holotype: A formalin fixed specimen, TNS-AL-57781 in TNS, from cultured strain PTG.

Type strain: PTG maintained in the Lake Biwa Environmental Research Institute, Japan.

Type locality: Lake Biwa, Shiga Pref., Japan.

Habitat: Plankton in lakes.

Pseudanabaena foetida var. ***subfoetida*** (Niiyama et Tuji) Tuji et Niiyama **comb. nov.**

Basionym: *Pseudanabaena subfoetida* Niiyama et Tuji in Niiyama *et al.*, Fottea **16**: 5. *f. 10*, *11*, *15*. 2016. (Fig. 1B)

Holotype: A formalin fixed specimen, TNS-AL-58650 in TNS, from cultured strain Ak1318 (PS1306) maintained in the Department of Botany, National Museum of Nature and Science.

Type locality: Lake Kasumigaura, Ibaraki Pref., Japan.

Habitat: Plankton in lakes.

Pseudanabaena foetida var. ***intermedia*** Tuji et Niiyama, **var. nov.** (Fig. 1C)

Description: Trichomes solitary, straight, bright blue-green colored, with conspicuous constrictions at cross-walls, 1.6–2.2 μm wide, without mucilage or sheath, not attenuated nor differentiated at the ends, without calyptra, infrequently move. Cells cylindrical with rounded ends, bright blue-green, longer than wide, 3.0–8.3 μm long, ratio of width to length ca. 1.6–4.6, with aerotopes at both ends of cells, differentiated in centro- and chro-matoplasmic regions. Cell division perpendicular to the longitudinal axis of a trichome. Trichomes separate between two neighboring cells or by fragmentation without necridic cells. Heterocytes are not known. Thallus has an extreme musty odor that comes from 2-methyl-isoborneol.

Holotype: A formalin fixed specimen, TNS-AL-58658 in TNS (Department of Botany, National Museum of Nature and Science), from cultured strain NIES-512.

Type strain: NIES-512 maintained in National Institute for Environmental Studies (NIES).

Type locality: Nagoya castle, Aichi Pref., Japan.

Habitat: Plankton in lakes and ponds.

Synonyms:

Phormidium tenue Gomont *sensu* N.Yamada in Watanabe *et al.* (2000).

Pseudanabaena galeata Böcher *sensu* T. Honma in Kasai *et al.* (2009).

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